

RESEARCH ARTICLE

Quadrupedal gaits in hexapod animals – inter-leg coordination in free-walking adult stick insects

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SUMMARY

The analysis of inter-leg coordination in insect walking is generally a study of six-legged locomotion. For decades, the stick insect *Carausius morosus* has been instrumental for unravelling the rules and mechanisms that control leg coordination in hexapods. We analysed inter-leg coordination in *C. morosus* that freely walked on straight paths on plane surfaces with different slopes. Consecutive 1.7 s sections were assigned inter-leg coordination patterns (which we call gaits) based on footfall patterns. Regular gaits, i.e. wave, tetrapod or tripod gaits, occurred in different proportions depending on surface slopes. Tetrapod gaits were observed most frequently, wave gaits only occurred on 90 deg inclining slopes and tripod gaits occurred most often on 15 deg declining slopes, i.e. in 40% of the sections. Depending on the slope, 36–66% of the sections were assigned irregular gaits. Irregular gaits were mostly due to multiple stepping by the front legs, which is perhaps probing behaviour, not phase coupled to the middle legs' cycles. In irregular gaits, middle leg and hindleg coordination was regular, related to quadrupedal walk and wave gaits. Apparently, front legs uncouple from and couple to the walking system without compromising middle leg and hindleg coordination. In front leg amputees, the remaining legs were strictly coordinated. In hindleg and middle leg amputees, the front legs continued multiple stepping. The coordination of middle leg amputees was maladapted, with front legs and hindlegs performing multiple steps or ipsilateral legs being in simultaneous swing. Thus, afferent information from middle legs might be necessary for a regular hindleg stepping pattern.

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INTRODUCTION

Obviously, the analysis of insect terrestrial locomotion is the analysis of hexapedal walking. Aside from some apparent specialists, e.g. praying mantis, mole cricket or locust, in insect imagines all three leg pairs mainly serve the purpose of walking. Consequently, descriptions of leg coordination during walking consider six legs. Just like legged animals in general, hexapeds use different inter-leg coordination patterns during walking to meet different behavioural demands.

Commonly, inter-leg coordination patterns are grouped into gaits. Insect gaits range from a tripod coordination in fast walkers to a metachronal or wave gait in slow walkers. Between these extremes, intermediate gaits occur (Hughes, 1952) (for reviews, see Wilson, 1966; Graham, 1985; Delcomyn, 1981; Ritzmann and Büschges, 2007). In the tripod gait, 'two mirror-image tripods step in an alternating pattern such that the animal always has at least three feet touching the ground' (Bender et al., 2011). In other words, in a tripod gait three legs swing together (Cruse et al., 2009). When insects walk slowly, a pattern is often observed in which only one leg swings together with a leg located diagonally on the other side. Here, the term tetrapod gait is occasionally used and in this gait 'at least four legs are on the ground at any moment of time' (Cruse et al., 2009).

For insects, the concept of gaits is not without controversy (Cruse et al., 2009) because, under certain conditions, gaits do not appear to be separable. Wendler found a gliding coordination of leg movements in mounted adult stick insects, walking on a passive treadmill (Wendler, 1964; Wendler, 1965). Coordination ranged from metachronal waves alternating between the left and right side at very low speeds of walking to a tripod coordination at high speeds (7 cm s^{-1}). Dürr stated that 'gaits may not be a helpful concept for describing leg coordination in all walking arthropods' because gaits in mounted stick insects that walk straight on a Styrofoam sphere cannot be identified unequivocally as a result of considerable variation of stepping patterns over time (Dürr, 2005). Of course, insect inter-leg coordination patterns depend on the behavioural context and environmental conditions such as surface structure, slopes, orientation of the body or specifics of an experimental setup (e.g. Spirito and Mushrush, 1979; Delcomyn, 1981; Graham, 1985; Duch and Pflüger, 1995; Dürr, 2005; Gruhn et al., 2009; Bender et al., 2011). And thus, in contrast to walks on the sphere, Graham reported that free-walking adult stick insects (*Carausius morosus*) on a horizontal surface almost exclusively use a 'bi-quadruped'; that is, a tetrapod gait (Graham, 1972). These patterns appear to be regular. However, Graham mentions incidental occurrences of errors in the normal metachronal sequence; for example, extra protractions of a front leg during walking. In sharp

contrast to Graham is the notion by Cruse (Cruse, 1976) that stick insects (*C. morosus*) walking on a horizontal plane use their front legs mainly as sensors. However, this view of front leg function does not appear to have received further attention although it implies consequences for models of the control of hexapedal locomotion. We therefore attempted to deepen the insight into inter-leg coordination in adult untethered stick insects (*C. morosus*). Specifically, we wished to answer the following questions. Is the behaviour of front legs in principle different from that of the other legs? Does front leg behaviour change depending on the actual inter-leg coordination pattern and surface slope? If yes, what are the effects of amputation of a segment's legs on the coordination patterns of the remaining legs?

We show here that in horizontal surface walking the front legs often perform a multiple stepping or probing behaviour that is independent of the adjacent legs' walking cycles and that is not seen in the other legs. Inter-leg coordination patterns and the occurrence of multiple stepping depend on surface slopes. The regularity of middle leg and hindleg coordination is not compromised by front leg multiple stepping or probing. Amputation of front leg or hindleg pairs has an impact on inter-leg coordination but not on the regularity of middle leg and hindleg coordination or multiple stepping behaviour in front legs. In contrast, amputation of middle leg pairs severely hampers the formation of a functional walking pattern in front legs and hindlegs. We conclude that front legs can be coupled to or decoupled from the locomotor system to generate multiple stepping or contribute to regular hexapedal walking. Furthermore, our data imply that middle leg stepping is a robust behaviour that contributes to the coordination of hindleg stepping. The different functionality of legs and the resulting flexibility of the walking system need therefore to be considered in modelling studies of insect locomotion.

MATERIALS AND METHODS

Experiments were performed with adult female stick insects *C. morosus*, Sinety 1901, from a colony maintained at the University of Cologne. The walking behaviour of seven to nine animals was investigated under different walking conditions. Animals were filmed while walking on a plain black fabric surface (160×90 cm). A white 18 mm tape was attached along the mid-line of this arena and a white board with a black stripe was positioned at the end of the arena to give animals orientation for straight walks. In addition, the white stripe served as a reference to determine walking direction. Only straight walks were used for evaluation. Animals that did not start walking voluntarily were briefly touched on the abdomen to trigger walking. To avoid the potential effect of this touch on the walking pattern, the first four steps of those walking sequences were excluded from the analysis. To obtain a 15 or 90 deg slope, the arena was elevated on one side.

Stick insects were filmed from above with an AVT Pike Camera (Allied Vision Technologies, Stadtroda, Germany) at 60 frames s⁻¹. The camera was mounted on a jointed articulated boom stand that allowed the moving insect to be followed continuously. The setup was illuminated by a halogen lamp. The camera was controlled by an AVT-Active-Cam-Viewer (Allied Vision Technologies; configurations: 640×480, monochrome, 8-bit; brightness 36, shutter 250, sharpness 2, digital zoom 1000, 60 frames s⁻¹). For recording, the lens was set to 8 mm and the aperture to f-number 5.6. Movies were analysed frame by frame using AVI edit (AM Software).

Identification of coordination patterns

To identify coordination patterns or gaits, foothold pattern diagrams (e.g. Fig. 1) were constructed in Excel (Microsoft Office 2007) by identification of the posterior extreme position (PEP, lift-off) and

the anterior extreme position (AEP, touch-down). Black bars indicate the swing phase of a leg. Frame by frame analysis accounts for an error of ±1 frame (i.e. 16.6 ms) when determining PEP and AEP. Sequences of continuous walking were segmented into sections of 100 frames (1.7 s) to determine a gait for each section. An alternative approach in which 100 frame sections were moved step by step yielded no difference in results and was not applied. We continued with the segmentation of sections of 100 frames. A sequence contained a mean of seven sections. Depending on walking speed, each section contained two to five steps by each leg.

A gait could be assigned to each section of 100 frames. To determine gaits, phase relationships were calculated as the onset of swing with respect to the stepping period of the right middle leg (R2) or right hindleg (R3) in the case of amputated middle legs. Fig. 1 shows idealized step patterns (see also Wilson, 1966) for a tripod gait in which three legs swing in synchrony (Fig. 1A) and two types of tetrapod gait – mirror images of one another – in which two diagonal legs swing in synchrony (Fig. 1B,C). These ideal patterns result in phase relationships of leg movements as given in Table 1. In our experiments, however, we never observed perfectly synchronous swing movements in either gait. Therefore, we tolerate a deviation from ideal phase relationships during swing by ±0.12. When assigning a gait, we allowed one erroneous step of a single leg per section.

In some experiments, a pair of front legs, middle legs or hindlegs was amputated at the coxa-trochanteral joint. To quantify the resulting quadrupedal gaits in front leg or hindleg amputees, phase relationships of the remaining legs were calculated with respect to the stepping period of the right middle leg (R2). In middle leg

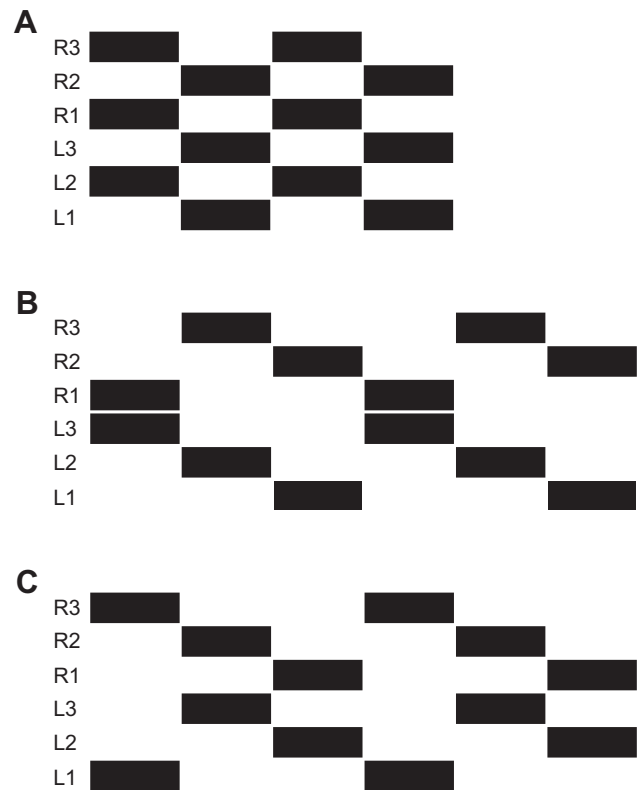


Fig. 1. Schematic drawing of different stereotypic hexapedal walking patterns. Black bars indicate leg swing. In tripod gait, three legs swing in synchrony (A), while in tetrapod gait, two diagonal legs swing synchronously (B,C).

Table 1. Stereotypic phase relationships of all legs within a cycle period of the right middle leg (R2) or the right hindleg (R3) for the different gaits

	R2				R3			
	Tetrapod 1	Tetrapod 2	Tripod	Circular mean	Tetrapod 1	Tetrapod 2	Tripod	Circular mean
R3	0.66	0.66	0.5	0.61	–	–	–	–
R2	–	–	–	–	0.33	0.33	0.5	0.34
R1	0.33	0.33	0.5	0.34	0.66	0.66	0	0.73
L3	0.33	0	0	0.14	0.66	0.33	0.5	0.52
L2	0.66	0.33	0.5	0.52	0	0.66	0	0.91
L1	0	0.66	0	0.88	0.33	0	0.5	0.26

Right (R) and left (L) legs are designated 1 (front) 2 (middle) or 3 (hind). Gaits were tetrapod 1, tetrapod 2 and tripod. Circular means of phases of the individual legs obtained in our experiments are also given.

amputees, the right hindleg (R3) was taken as the reference. Four different quadrupedal gaits were observed: in trot gait, two diagonal leg pairs always swing together (Fig. 2A); in the two walk gaits (canter gait coordination), synchronous swing of a diagonal pair is followed by two single leg swing phases (see Fig. 2B,C for walk 1 and 2, respectively); and in wave gait, only a single leg swings (Fig. 2D).

It was not always possible to assign a gait to a section. These cases were due to irregularities, like a continuous gait transition or multiple steps by legs; for example, ‘probing’ of front legs. A section with such irregularities was classified as irregular gait (see Fig. 3C and Fig. 5A for typical examples). To determine the frequency of occurrence of gaits and corresponding phase relationships, data from different animals were pooled. Before pooling, data were weighted according to the number of walking sequences that were performed by each animal.

Walking speed

Walking speeds of intact and amputated animals were evaluated for sections with StickTracker, a customized Matlab program (The MathWorks, Inc., Natick, MA, USA) by Dr Till Bockemühl. StickTracker calculates velocity by frame-to-frame movements of the point defined by the intersection of the lateral axis through both hindleg coxae and the longitudinal axis in relation to ground markers.

Statistics

Circular statistics were performed using the circular statistics toolbox for Matlab (The MathWorks, Inc.) (Berens, 2009). The Rayleigh test (Batschelet, 1981) was used to test whether phases were randomly distributed or whether a predominant directionality is present. The Watson–Williams *F*-test (Batschelet, 1981) was used to test for differences in length of the mean resulting phase vectors. The length of the mean resulting vector is a crucial quantity for the measurement of circular spread. The closer it is to one, the more concentrated the data sample is around the mean direction. This test was performed using ORIANA 4 (Kovach Computing Services, Anglesey, UK). For the statistical evaluation of the phases, all steps within a sequence were taken into account. To test whether the multiple steps of the front legs were randomly distributed in phase with respect to the stepping period of R2, only the sections in which multiple stepping occurred were considered.

Differences between the mean number of steps performed across all animals for different legs in different walking situations were tested in Matlab using a one-tail ANOVA (see Fig. 4E, Fig. 7B, Fig. 8B and Fig. 9B).

The occurrences of different gaits in different walking situations were compared and tested for significance using the Wilcoxon rank

sum test in Matlab (see Fig. 4A–D, pooled weighted data). In the experiments with animals with amputated legs, we put all regular gaits together (trot, walk 1, walk 2 and wave) and compared their occurrence with that of irregular gaits (Fig. 7A, Fig. 8B and Fig. 9B). The Wilcoxon rank sum test in Matlab was also used to determine differences in walking speed between groups.

RESULTS
Hexapedal walking

Stick insects (*C. morosus*) that walked a straight path on a horizontal surface adopted a tetrapod gait in 43.7% of 32 sections from 9 animals. A typical example of a tetrapod gait section is shown in Fig. 3B. Generally, both mirror-image tetrapod gaits were used by the animals (Fig. 1B,C; see Materials and methods for details).

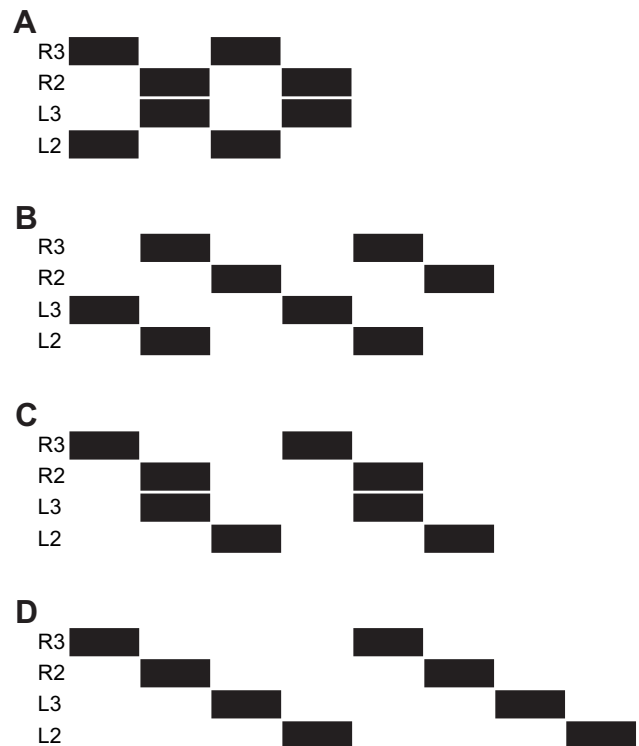


Fig. 2. Schematic drawing of different stereotypic quadrupedal walking patterns. In trot, two diagonal legs swing in synchrony (A). In walk, synchronous swing of a diagonal pair of legs is followed by two single leg swing phases (B,C) and in wave gait, only a single leg swings (D). The stereotypic patterns are shown for the case of stick insect front leg amputees.

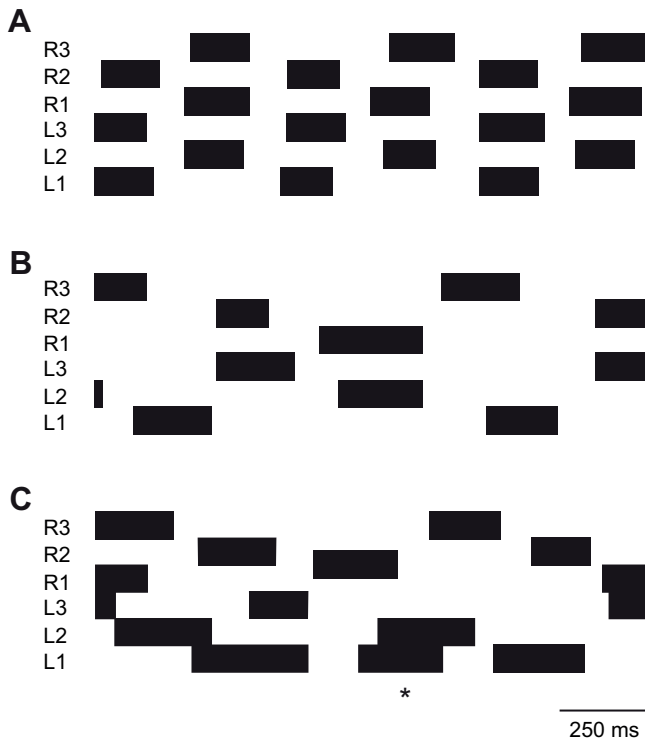


Fig. 3. Examples of tetrapod (A), tripod (B) and irregular (C) walking patterns observed in the adult stick insect. *Simultaneous swing of adjacent legs.

Animals adopted the tripod gait significantly less often (16.6%) (Fig. 3A). Occasionally, an animal switched gaits within a walking sequence. These switches were not abrupt but rather smooth transitions.

The occurrence of regular gaits was reduced in upward slope walking. On a 15 deg upward slope, tetrapod gait was adopted in 32.1% and tripod gait in 8.7% of all sections ($N=8$ animals, $n=36$ sequences of continuous walking). On a 90 deg upward slope, tetrapod gait was adopted in 28.1% and tripod gait in 5.7% of the sections ($N=7$, $n=21$). Only on the 90 deg slope did animals occasionally use the wave gait (9% of sections). In all three walking situations, a tetrapod gait was adopted significantly more often than a tripod gait. In contrast to this, in downward slope walking the relative number of tripod gait sections increased as tetrapod gaits were observed in 23.9% and tripod gaits in 40.3% of sections ($N=7$, $n=36$; differences between occurrences of gaits were not significant, $P>0.05$).

Besides the regular tetrapod and tripod gaits, we frequently observed irregular non-stereotypic walking patterns in all four walking conditions (example footfall patterns are shown in Fig. 3C, Fig. 5A). These irregular walking patterns, or irregular gaits, occurred even though we allowed a certain variability when assigning a tetrapod or tripod gait to a section (see Materials and methods for details). Irregular gaits occurred in 39.7% of sections recorded on the horizontal surface, and in 35.8% in walks on the 15 deg downward slope. They occurred more often in upward slope walking: in 56.4% and 66.3% of the cases on 15 and 90 deg slopes, respectively. Data are summarized in Fig. 4A–D (grey bars). Closer inspection of irregular gait sections revealed that, on a horizontal surface, the number of steps, i.e. the swing movements, performed by the front legs (R1, $n=200$; L1, $n=253$), was significantly higher

($P<0.05$) than the number of steps by the hindlegs (R3, $n=149$; L3, $n=156$) or middle legs (R2, $n=156$; L2, $n=152$; Fig. 4E). Step numbers of the hindlegs and middle legs were not significantly different ($P>0.05$). Similarly, in slope walking, the front legs performed significantly more steps than the middle legs or hindlegs ($P<0.001$) (Fig. 4E). Therefore, if no switching between the different gaits occurred, inconsistencies in irregular gaits were mainly due to more frequent stepping of the front legs. Sometimes, the animals showed rocking behaviour during walking (up to 22% on the horizontal surface and less in the other walking situations). Rocking behaviour is a side-to-side movement usually performed by stick insects that do not locomote. Occasionally, this behaviour is apparent in animals approaching the end of a walk (Pflüger, 1977). Rocking behaviour was equally distributed among gaits. We therefore conclude that rocking behaviour does not affect the distribution of the occurrence of the different gaits.

When ignoring front legs in the analysis of irregular gait sections, more regular stereotypic walking patterns of the middle legs and hindlegs became obvious. Leg coordination was comparable to that of the quadrupedal gaits walk and trot (Fig. 2, see Materials and methods for details). Fig. 5A shows such a regular middle leg and hindleg walk-like pattern (black bars) with irregular stepping of the front legs (grey bars). Phases of multiple steps of both front legs were randomly distributed with respect to the reference leg (R2 and R3) cycles. Mean direction vectors in circular plots (Fig. 5B; data not shown for reference leg R3) did not indicate a significant directionality (Rayleigh test). The relative occurrence of quadrupedal gaits on the horizontal surface with a walk-like pattern was 76.3% (7% wave gait), with a trot-like pattern was 17.3%, and with an irregular pattern was 6.4% ($N=9$, $n=32$); on a 15 deg upward slope the values were: walk-like 67.5%, trot-like 5.5%, irregular 27% ($N=8$, $n=36$); on a 90 deg upward slope the values were: walk-like 75.9%, trot-like 0.5%, irregular 23.6% ($N=7$, $n=21$); and on a 15 deg downward slope the values were: walk-like 42.8%, trot-like 43.3%, irregular 13.9% ($N=7$, $n=36$). The relative occurrence of different gaits with and without the front legs in the different walking situations is illustrated in Fig. 4A–D. The occurrence of irregular gaits significantly decreased when ignoring the front legs in walks on a horizontal surface and on upward slopes. The remaining irregular gaits were mainly due to gliding transitions between gaits. In contrast to irregular gaits, the relative occurrence of walk-like gaits was significantly higher than the amount of the corresponding tetrapod gaits in all walking situations except for downward slopes. The relative occurrence of the trot-like and corresponding tripod gait was comparable in all four walking situations. Wave gaits only occurred in 90 deg upward slope walks.

Walking in leg amputees

Multiple stepping performed by the front legs during walking suggests that front legs not only function as locomotor organs but also serve an additional function, for example to probe the environment (Cruse, 1976). Because this function is performed during walking, we were interested in whether front legs are necessary at all for the establishment of a regular gait in middle legs and hindlegs. We were also interested in whether front legs assume a more regular walking pattern in animals that lack support by either both middle legs or both hind legs. Therefore, we amputated leg pairs and subsequently allowed the animals to walk on a horizontal surface. From these walks we determined gaits, and calculated phases of the individual legs with respect to R2 or R3 cycles.

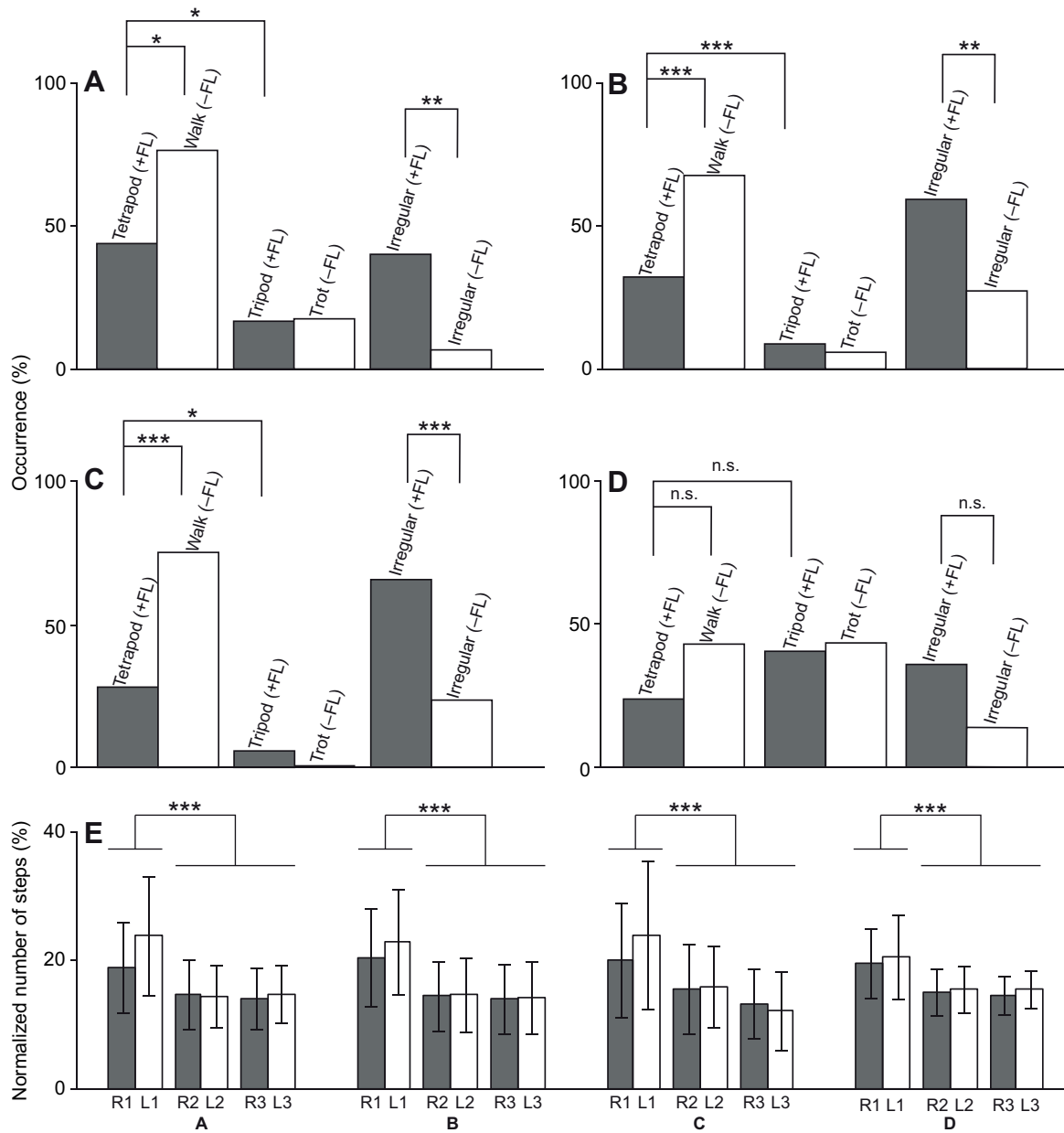


Fig. 4. Frequency of occurrence of different gaits in the different walking situations: (A) horizontal surface; (B) 15 deg upward slope; (C) 90 deg upward slope; and (D) 15 deg downward slope. Grey bars indicate hexapedal gaits; +FL, front legs were considered when assigning a gait. White bars indicate quadrupedal gaits; -FL, front legs were not considered when assigning a gait. Significance in the difference of occurrence of the different gaits was tested using the Wilcoxon rank sum test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant). The tetrapod gait was performed significantly more often than the tripod gait in A-C. When ignoring the front legs, the occurrence of irregular gaits significantly decreased in comparison to when all legs were considered. In parallel, the occurrence of walks when front legs were ignored was significantly higher than the occurrence of tetrapod gait when all legs were considered (A-C). (E) Normalized number of steps by the different legs in A-D. The front legs R1 and L1 performed a significantly greater number of steps than the other legs in all four walking situations (*** $P < 0.001$). Significance was tested using a one-tail ANOVA.

Phase relationships in intact stick insects

As a reference for the phase relationships between legs in amputation experiments, we determined the phases of swing movements in intact animals that walked on the horizontal surface. Phases of L3, L2, R3 and R1 were calculated with respect to the stepping period of R2 [$\Phi(R2-L3)$, $\Phi(R2-L2)$, $\Phi(R2-R3)$ and $\Phi(R2-R1)$, respectively] and phases of L3, L2, R2 and R1 with respect to the R3 period [$\Phi(R3-L3)$, $\Phi(R3-L2)$, $\Phi(R3-R2)$ and $\Phi(R3-R1)$, respectively]. The results are shown in Fig. 6. Animals used a tetrapod gait in 43.7% of the sections (see above). The two mirror-image tetrapod

gaits were used equally often. When the front legs were ignored, 76.3% of the sections were classified as walk-like gaits (see above). Therefore, we expected phases to be most frequently near $\Phi(R2-L3)=0$ and 0.33, $\Phi(R2-L2)=0.33$ and 0.66, $\Phi(R2-R1)=0.33$ and $\Phi(R2-R3)=0.66$ and at $\Phi(R3-L3)=0.33$ and 0.66, $\Phi(R3-L2)=0$ and 0.66, $\Phi(R3-R1)=0.66$ and $\Phi(R3-R2)=0.33$ (see also stereotypic phases in Table 1).

When R2 was the reference leg, phases had the following circular means: $\Phi(R2-L3)=0.14$ ($n=407$), $\Phi(R2-L2)=0.52$ ($n=418$), $\Phi(R2-R1)=0.34$ ($n=474$) and $\Phi(R2-R3)=0.61$ ($n=408$)

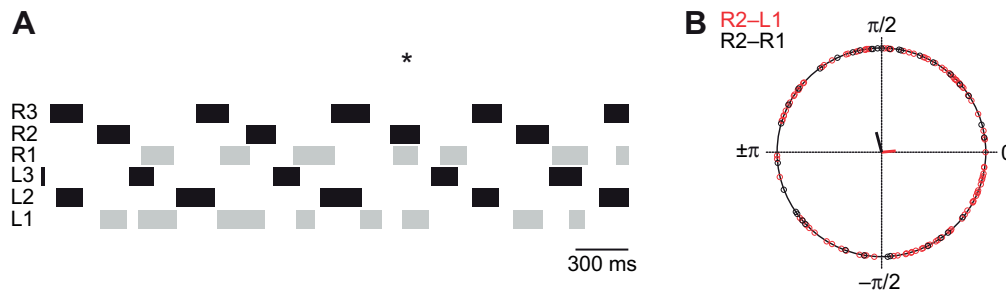


Fig. 5. (A) Example of an irregular walking pattern observed in an adult stick insect walking on a horizontal surface. Middle legs and hindlegs are coordinated in a regular quadrupedal walking pattern. Grey bars indicate that front legs were not considered. (B) Phase distributions of R1 (black) and L1 (red) in the R2 stepping cycle, only for irregular walking patterns. Phases for both front legs are randomly distributed and the resultant mean vectors (black and red) show no significant directionality (in both cases $P > 0.05$). *Simultaneous swing of adjacent legs.

($N=9$; Fig. 6A, Table 1). The data meet the expectations as phases for L3 and L2 lie well within the two phases that these legs adopt in an ideal tetrapod gait, and the phases of R3 and R1 are very close to their expected values. In addition, as expected, distributions for L3 and L2 phases were broader than those for R3 and R1 (Fig. 6A). This observation was corroborated by calculating the length of the mean direction vectors for the four legs. Vectors for L3 and L2 were significantly shorter than vectors for R3 and R1 ($P < 0.001$, data not shown). The data generally met the expectations when R3 was the reference leg. Circular means of the phases were $\Phi(R3-L2)=0.91$, $\Phi(R3-L3)=0.52$, $\Phi(R3-R1)=0.73$ and $\Phi(R3-R2)=0.34$ ($n=417$). Also, a broader distribution for L3 and L2 phases in comparison to R2 and R1 phases was indicated by the significantly different lengths of the mean direction vectors ($P < 0.001$, data not shown; see also Fig. 6B).

Gaits and phase relationships in front leg amputees

It is striking that front leg amputees almost exclusively used regular gaits (Fig. 7A). Irregular gaits were observed in only in 5.7% of the sections ($N=7$, $n=161$). Usually, animals walked with a wave gait (61%). Less often, the two mirror-image walk gaits were observed (20% walk 1, 18% walk 2; see Fig. 2 for ideal footfall patterns). Rarely, animals walked with trot gait (1%). The average number of steps (14–19 steps per leg) by individual legs in irregular gait sections did not significantly differ (Fig. 7B, $P > 0.05$).

The circular means of the phases of R3 and L3 with respect to the R2 step cycle were $\Phi(R2-R3)=0.73$ ($n=202$) and $\Phi(R2-L3)=0.22$ ($n=202$), respectively (Fig. 7C). This was expected as they are close to the ideal phases in the observed quadrupedal gaits [$\Phi(R2-R3)=0.66$ (walk) or 0.75 (wave gait) and $\Phi(R2-L3)=0$ and 0.33 (walk) or 0.25 (wave gait); see Fig. 2B–D]. Also, the phase relationship $\Phi(R2-L2)=0.47$ was as expected for a typical wave gait (not shown).

To compare the phase distributions of the left and right hindleg (L3, R3) with respect to the R2 cycle in intact animals and in front leg amputees, we calculated mean resultant phase vectors and their lengths (Fig. 7D,E). All four vectors had significant directionality (Rayleigh test; $P < 0.001$). Mean direction vectors of phase distributions of L3, L2 and R3 with respect to the R2 cycle in front leg amputees were significantly longer than corresponding vectors from intact animals ($P < 0.05$; Fig. 7D,E). This means that phase distributions of L3, L2 and R3 with respect to the R2 cycle become narrower and thus more distinct in front leg amputees (compare also green and blue distributions in Fig. 6A and Fig. 7C).

Gaits and phase relationships in hindleg amputees

In hindleg amputee walking, the percentage of irregular gaits was about three times that of front leg amputee walking, i.e. 17.2% ($N=8$, $n=30$; Fig. 8A); 42% of the regular gait sections were wave gaits and 58% were walk gaits (40% walk 1, 18% walk 2). Trot was not observed. In irregular gaits, the number of front leg steps was significantly higher than the number of middle leg steps ($P < 0.001$); 28% of all leg steps were L1 steps, 34% were R1 steps, 20% were L2 steps and 18% were R2 steps (Fig. 8B). This result indicates that irregular gaits are mainly due to multiple stepping or probing of the front legs as observed in intact animals.

The circular means of the phases of L2 and R1 with respect to the R2 cycle were $\Phi(R2-L2)=0.52$ ($n=253$) and $\Phi(R2-R1)=0.25$ ($n=344$) (see grey and red distributions in Fig. 8C). These phases were comparable to respective phases in the intact animal (compare with Fig. 6A). Mean phase vectors from hindleg amputees and intact animals had a significant directionality (Rayleigh test; $P < 0.001$). Mean direction vectors of phase distributions of L2 and R1 with respect to the R2 cycle, however, were not significantly longer than corresponding vectors from intact animals ($P > 0.05$; Fig. 8D,E). The phase distribution of L2 was significantly broader in hindleg amputees than in front leg amputees ($P < 0.001$, data not shown). Thus, the distribution of the phases of L2 and R1 with respect to the R2 cycle did not become narrower and thus more distinct in hindleg amputees (compare also distributions for L2 and R1 in Fig. 6A and Fig. 8C).

In hindleg amputees, the abdomen was on the ground in 67.5% and in the air in 32.5% of the sections. However, the percentage of irregular sections was not significantly different between conditions (17.5% in animals with abdomen on the ground and 16.9% in animals with abdomen in the air). Thus, the position of the abdomen does not appear to affect the additional stepping activity in front legs.

Gaits and phase relationships in middle leg amputees

The percentage of irregular gaits was 27.7% in middle leg amputees ($N=7$, $n=47$; Fig. 9A); 56% of the regular gait sections were wave gaits and 44% were walk gaits (27% walk 1, 16% walk 2). Only 1% of all regular walking sections were trot. Interestingly, the average step number of individual legs in irregular gait sections did not significantly differ (Fig. 9B). Closer inspection revealed that, in contrast to all walking situations described above, irregular gaits did not mainly result from multiple front leg stepping. We observed multiple front leg steps in 22% of the irregular gait sections. In 12%, we observed multiple hindleg steps that were never observed in our other experimental situations. Of the irregular gait sections, 14%

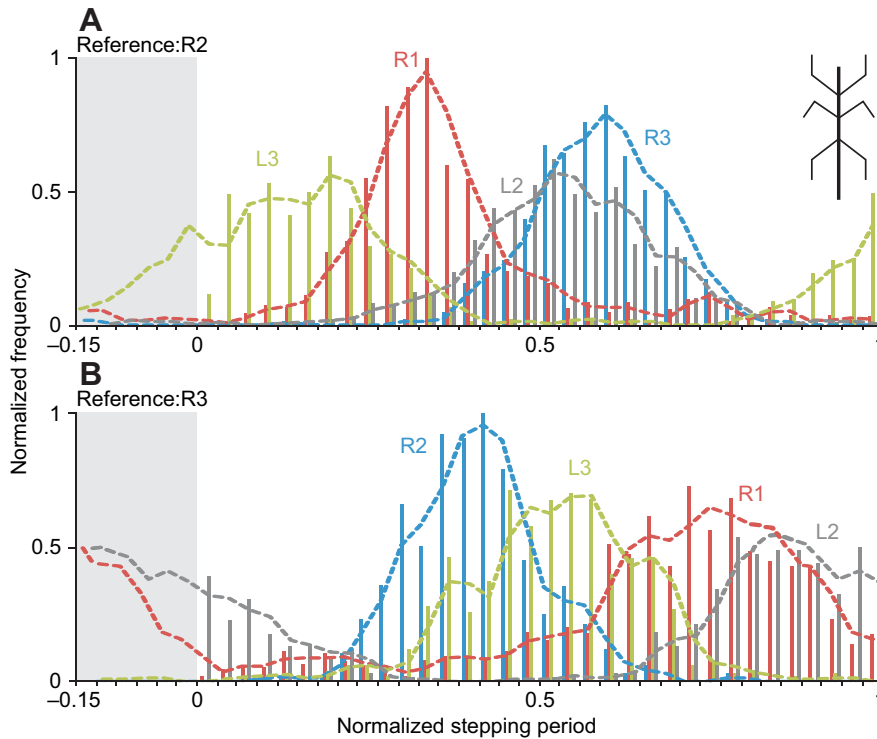


Fig. 6. Phases in intact animals walking on a horizontal surface. (A) Phase distributions of the right front leg (R1, red), right hindleg (R3, blue), left middle leg (L2, grey) and left hindleg (L3, green) with respect to the phase of the right middle leg (R2). (B) Phase distributions of the right front leg (R1, red), right middle leg (R2, blue), left middle leg (L2, grey) and left hindleg (L3, green) with respect to the phase of the right hindleg (R3).

were due to fast, simultaneous protraction of three legs (R3, L3, L1 or L3, R3, R1) and 16% to simultaneous swing movements of the two ipsilateral legs (R3, R1 or L3, L1; see supplementary material Movies 1–6). In 36% of irregular gait sections, switching between gaits or other irregular patterns occurred.

In this walking situation, we used R3 as the reference leg to calculate the phases of the three remaining legs. The circular means of the phases of R1, L3 and L1 with respect to the R3 cycle were $\Phi(R3-R1)=0.31$, $\Phi(R3-L3)=0.51$ and $\Phi(R3-L1)=0.76$ (see Fig. 9C; note, only distributions of R1 and L3 are shown). As expected from the predominant quadrupedal wave and walk gaits (see above), the R1 swing starts far earlier in the R3 cycle than in the intact animal [$\Phi(R3-R1)=0.73$; compare with Fig. 6B]. The respective mean direction vectors clearly indicate the difference in phase (Fig. 9D). Mean phase vectors from middle leg amputees and intact animals had a significant directionality (Rayleigh test; $P<0.001$; Fig. 9D,E).

The lengths of the mean direction vectors of R1 and L3 phase distributions with respect to the R3 cycle differed significantly between middle leg amputees and intact animals ($P<0.05$). The distribution of the R1 phases with respect to the R3 cycle became narrower in middle leg amputees (mean direction vector is longer). In contrast, the L3 phase distribution became broader, as indicated by the much shorter vector (compare the red and black vector in Fig. 9E).

To investigate whether irregular walking is primarily a consequence of a reduction in walking speed, we compared walking speeds in intact animals and middle leg amputees. As shown in Fig. 10, in intact animals (red box plots, $N=9$), the velocities of irregular ($n=59$) and tetrapod ($n=76$) gaits were not significantly different. The velocities of animals using these two gaits were significantly slower than the velocities of animals using tripod ($n=29$) gait. In middle leg amputees (black box plots, $N=7$), the velocities of animals using irregular ($n=81$), walk/wave ($n=168$) or trot ($n=4$) gaits did not differ significantly. Middle leg amputees

that used irregular and walk/wave gaits were significantly slower than intact animals using irregular and tetrapod gaits. Thus, irregular gaits occur at slow velocities. However, not all animals that walk slowly necessarily use an irregular gait.

DISCUSSION

We investigated the leg coordination patterns of stick insects (*C. morosus*) walking freely along a straight path on a plane horizontal surface as well as on inclining and declining surfaces. We showed that, on horizontal and on inclining surfaces, tetrapod gaits occur significantly more often than a tripod walking pattern. On the horizontal surface, for example, tetrapod gaits occurred about 2.6 times more often than a tripod gait. On the 15 deg declining surface, there was no significant difference between the occurrence of tripod and tetrapod gaits. While Graham (Graham, 1972) states that a tripod gait is relatively rare in adult stick insects (he does not give quantitative data), our data show that the occurrence of a tripod gait in adult stick insects is not generally rare but rather context dependent. Generally, gaits in animals are correlated with walking speed (Alexander, 1989). It is thus reasonable to assume that the higher probability of tripod gaits on the declining surface is due to an increase in speed. This conclusion is supported by our observation that tripod gaits occur at higher speeds and by a previous finding that in stick insects walking on a treadmill, phase relationships of different legs change in gliding coordination as a function of walking speed (Wendler, 1965). In Wendler's experiments, a phase relationship corresponding to a tripod gait was obtained at higher walking speeds than those for the phase relationship corresponding to a tetrapod gait. Our results from walking on slopes suggest that load conditions may matter in determining gait. Nothing is known about this in stick insects yet. However, it has been reported in the locust (Duch and Pflüger, 1995) that 'motor patterns are relatively constant for a given walking situation, but are markedly altered under different conditions, such as horizontal walking, vertical climbing and upside down walking'.

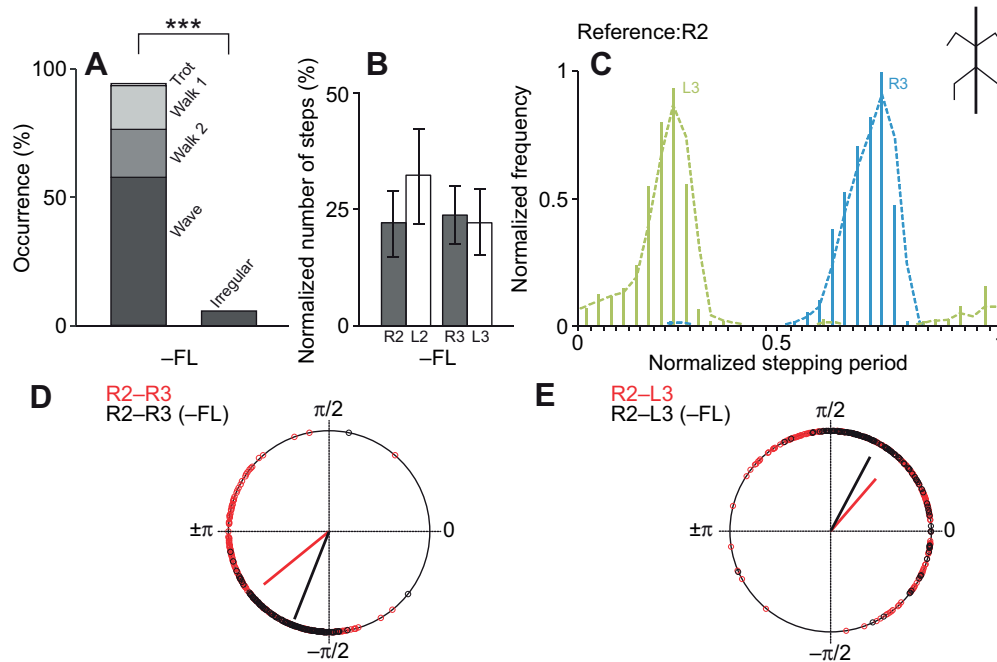


Fig. 7. Frequency of occurrence of different gaits (A), normalized number of steps by the middle legs and hindlegs (B), and phase distribution of legs (C–E) in front leg amputees. (A) Normalized occurrence of regular gaits (trot, walk 1, walk 2 and wave; left bar) and irregular gaits (right bar), which are rare occurrences. Within the regular gaits, the wave gait is the most common ($***P < 0.001$). The two walk patterns (1 and 2) are used equally often. (B) There is no significant difference between the number of steps of the hindlegs and middle legs. (C) Phase distributions of the right hindleg (R3, blue) and the left hindleg (L3, green) with respect to the phase of R2. (D, E) Phase distributions of R3 and L3 for intact animals (red) and front leg amputees (black, –FL) shown as circles on the unit circle. Red and black lines indicate the direction and magnitude of the mean resultant vector in intact animals and amputees, respectively. In all four cases, significant predominant directionality of the mean resultant vectors is given ($P < 0.001$). Mean resultant vectors for front leg amputees (black in D and E) are significantly longer than those for intact animals (red in D and E; $P < 0.05$). These data indicate that distributions become narrower if the front legs are amputated.

Another interesting finding of our experiments is that animals often performed ‘irregular’ walking patterns that drastically differed from the classical gaits. These irregular gaits were most often observed on inclining slopes. On the horizontal surface, 39.7% of walking sections qualified as irregular gaits. In general, these gaits were a result of the occurrence of multiple front leg steps in the reference leg’s (usually R2) cycle. A small amount of coordination irregularity is due to transitions between gaits. Our finding does not concur with that of Graham (Graham, 1972), who observed only eight ‘extra’ protractions in 400 leg cycles in adult stick insects walking on a horizontal surface for both front legs. However, our data are corroborated by Cruse’s observation (Cruse, 1976) that *C. morosus* walking on a horizontal plane ‘often make groping movements’. Cruse concluded, ‘In a walk on the horizontal plane: the forelegs mainly have feeler function’. Judging from our data, this statement appears to be a slight exaggeration. However, the appearance of front leg multiple stepping is indeed context dependent. In our experiments, for example, the appearance of irregular coordination patterns increased during upward slope walking (Fig. 4), and Cruse (Cruse, 1976) mentions that front legs are much more regularly moved when animals walk a 30 mm wide horizontal path. We will avoid using the term feeler, although the behaviour suggests that the legs are used for probing the ground. Multiple stepping or probing is worthy of receiving further attention. First, stick insect legs do perform stereotypic searching movements in the context of loss of ground contact when reaching a gap (Dürr, 2001), although we do not know whether the multiple stepping that we observed is related to searching. Second, afferent feedback during

ground contact in probing behaviour is likely to be different from feedback during ground contact in stance phase, in which the leg supports the body or provides a propulsive force (Zill et al., 2012). Such feedback does matter for inter-leg coordination (Wendler, 1965) and may be different during irregular gaits *versus* regular ones.

The multiple front leg steps were never phase coupled to the reference leg’s cycle (Fig. 5B). When this behaviour occurred, middle legs and hindlegs continued to perform regular stereotypic walking patterns. These patterns relate to the two quadrupedal mirror-image walk gaits and the wave gait (Fig. 2B–D). It thus appears that the front legs can be uncoupled from the walking system without compromising the coordination of the other legs. The occurrence of front leg multiple stepping is limited to walk and wave gaits. This limitation is functional as tetrapod gaits with only one leg on each side in swing provide more stability during multiple stepping than a tripod gait.

Obviously, multiple stepping of the front legs during the step cycle of the right middle leg (R2) implies simultaneous protraction of ipsilateral front and middle legs (Fig. 3C, Fig. 5A). Cruse and co-workers identified six rules for leg coordination that operate between adjacent legs in the stick insect (Dean, 1989; Cruse, 1990). These rules do not consider multiple stepping of the front legs. In particular, rule 1, which establishes that swing prevents lift-off in the next anterior leg through forward-directed inhibition, is not always obeyed. However, context-dependent changes in the strength and efficacy of leg coordination mechanisms in stick insects have been described by Dürr (Dürr, 2005). Dürr concludes that ‘the coordination rules that are thought to underlie many adaptive

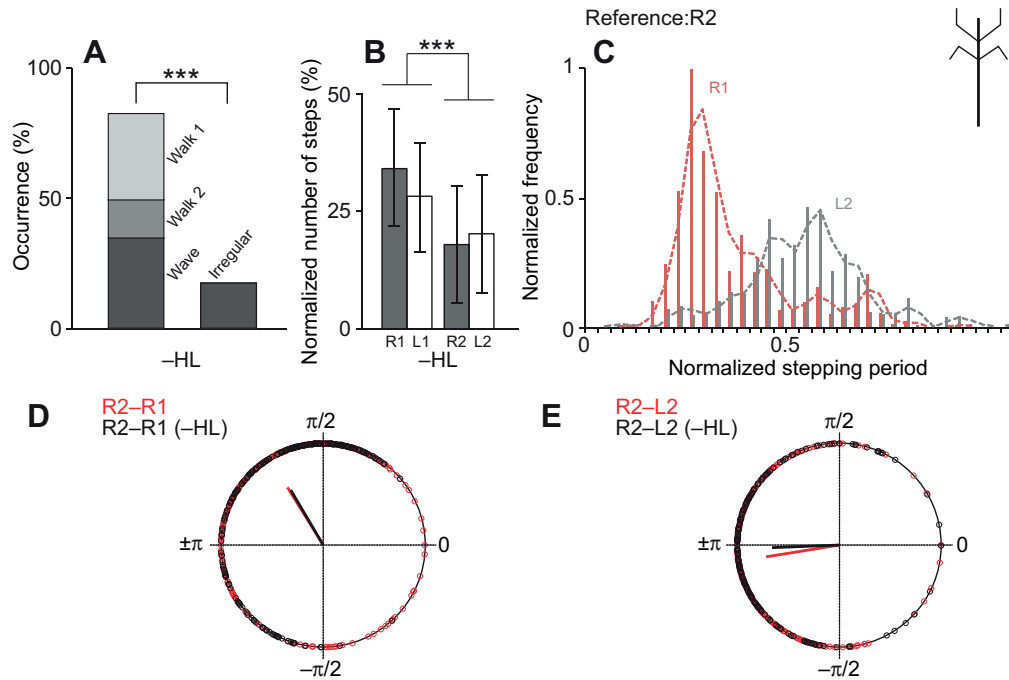


Fig. 8. Frequency of occurrence of different gaits (A), normalized number of steps by the front and middle legs (B), and phase distribution of legs (C–E) in hindleg amputees. (A) Regular gaits (walk 1, walk 2 and wave) are adopted significantly more often than irregular gaits (*** $P < 0.001$). No trot gait was observed. In most cases animals used the walk 1 and wave gait. (B) Normalized number of steps of the front and middle legs. Both front legs step significantly more often than the middle legs ($P < 0.001$). (C) Phase distributions for the right front leg (R1, red) and the left middle leg (L2, grey) with respect to the phase of R2. (D, E) Phase distributions of R1 and L2 for intact animals (red) and hindleg amputees (black, -HL). Red and black lines indicate the direction and magnitude of the mean resultant vector in intact animals and amputees, respectively. In all four cases, significant predominant directionality of the mean resultant vectors is given ($P < 0.001$). Mean resultant vectors for amputees (black in D and E) do not significantly differ in length from those for intact animals (red in D and E; $P > 0.05$). These data indicate that the distributions become neither broader nor narrower if the hindlegs are amputated.

properties of the walking system, themselves adapt in a context-dependent manner'.

The general layout of the insect nervous system appears to be well suited to allow functionally specialized legs to be uncoupled from or coupled to the locomotor system. For example, the praying mantis, which has front legs designed for catching prey, normally walks with its middle legs and hindlegs using wave or walk gaits, like the front leg-amputated stick insect. During fast walking, however, the front legs may be used as 'walking legs' as well, leading to a tetrapod or tripod gait (Roeder, 1937). In locusts walking on a flat surface, hindlegs that are specialized for jumping may step such that it is not possible to define a hexapod gait, or may be well coordinated with front and middle legs. However, a front leg and its contralateral middle leg are always precisely coordinated in-phase (Pearson and Franklin, 1984).

Intact animals versus front leg amputees

In front leg amputees, phase distributions of both hindlegs and the left middle leg with respect to the right middle leg were less broad than in the intact animal. Also, the number of steps performed by the remaining legs did not differ significantly from one another (Fig. 7). These results suggest that inter-segmental information from the front legs is not necessary to coordinate and stabilize regular walking in the middle legs and hindlegs. Rather, the presence of front legs appears to cause a weaker coupling of the middle legs and hindlegs. In addition, the onset of hindleg swing in the ipsilateral middle leg cycle of front leg amputees is shifted from 0.61 in the intact animal to 0.73 in amputees. This shift is due to the appearance of the wave gait.

We do not know whether front leg multiple stepping generates intersegmental signals that are transmitted from the prothoracic ganglion to the mesothoracic ganglion. Generally, single front leg stepping in stick insects is able to modulate the membrane potential of ipsilateral middle leg motoneurons: middle leg protractor and retractor motoneurons in the deafferented mesothoracic ganglion become rhythmically active and phase coupled to ipsilateral front leg stepping on a treadmill (Ludwar et al., 2005; Borgmann et al., 2007). Borgmann and colleagues (Borgmann et al., 2009) have shown that middle leg sensory signals from campaniform sensilla could overcome front leg step-induced entrainment. Such local dominance might also adjust the effects of potential intersegmental signals transmitted during front leg multiple stepping.

Intact animals versus hindleg amputees

In hindleg amputees, 17.2% of walking sections were assigned to the category of irregular gaits based on the occurrence of multiple front leg steps (Fig. 8A, B). This observation is surprising because front leg multiple stepping is likely to compromise stability in the four-legged animal. And after all, multiple front leg stepping appears to be a context-dependent and therefore modifiable behaviour.

As the phase distribution of the left middle leg (L2) with respect to the phase of R2 did not change significantly, the regularity of middle leg movements remained unchanged in hindleg amputees. We therefore suggest that regular stepping of the middle legs does not depend on inter-segmental information from the hindlegs. Although middle leg stepping is quite regular, there might be an effect of multiple front leg stepping on middle leg stepping because

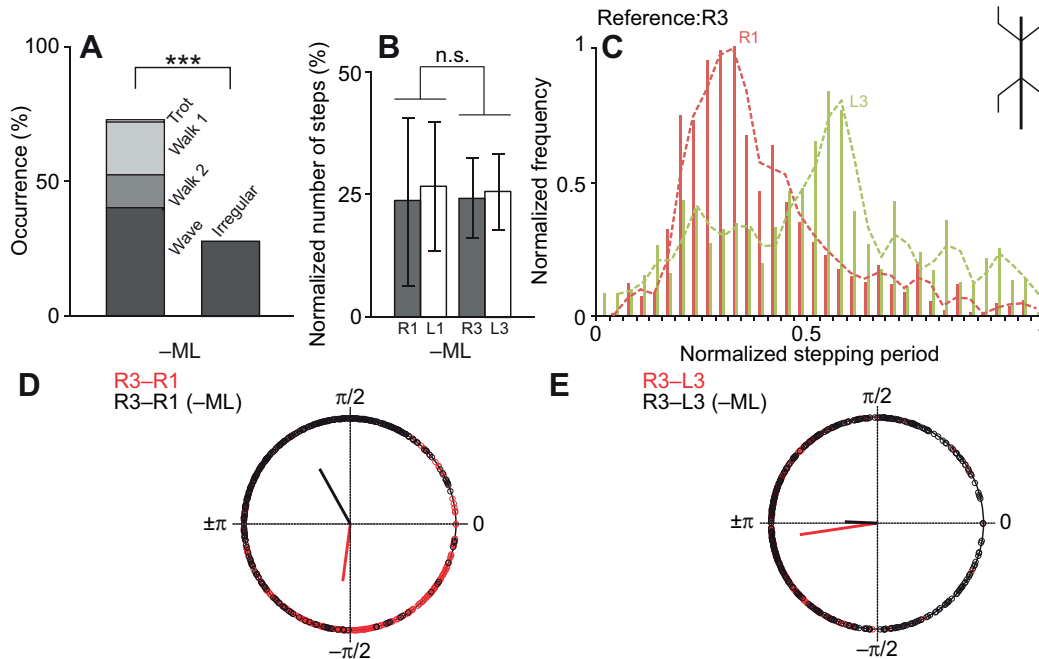


Fig. 9. Frequency of occurrence of the different gaits (A), normalized number of steps by the front legs and hindlegs (B), and phase distribution of the legs (C–E) in middle leg amputees. (A) Regular gaits (trot, walk 1, walk 2 and wave) are adopted significantly more often than irregular gaits (***) ($P < 0.001$). Animals mostly used the wave gait. (B) There is no significant difference between the normalized number of front and middle leg steps. (C) Phase distributions for the right front leg (R1, red) and the left hindleg (L3, green) with respect to the phase of R3. (D, E) Phase distributions of R1 and L3 for intact animals (red) and middle leg amputees (black, –ML). Red and black lines indicate the direction and magnitude of the mean resultant vector in intact animals and amputees, respectively. In all four cases significant predominant directionality of the mean resultant vectors is given ($P < 0.001$). The mean resultant vector of the phase distribution of L3 in the case of amputated middle legs (black in E) is significantly shorter than that for intact animals (red in E) ($P < 0.05$). Mean resultant phase vector for R1 is significantly longer in amputees than in intact animals ($P < 0.05$; D). These data indicate that the phase distribution of L3 with respect to the phase of R3 becomes significantly broader if the middle legs are amputated, whereas the broadness of the phase distribution of R1 decreases.

the L2–R2 phase distribution was broader in intact animals and hindleg amputees than in front leg amputees.

Intact animals versus middle leg amputees

In middle leg amputees, the percentage of irregular gait sections (~28%) was higher than in front leg or hindleg amputees. In contrast to all other cases described here, in middle leg amputees, multiple stepping of the front legs was not the main cause of irregular gaits. In 12% of all irregular sections, we observed multiple hindleg steps that almost never appeared in other conditions. Also, unstable coordination patterns occurred with both hindlegs and a front leg (14%) or two ipsilateral legs in swing phase (16%). Altogether, middle leg amputation destabilizes the walking system more than amputation of any other leg pair. A similar unstable coordination in middle leg amputees has been observed in cockroaches and in the grasshopper *Romalea* (Wilson, 1966). If regular gaits occurred in middle leg amputees, the wave gait and walk gaits were observed most often (Hughes, 1957; Wilson, 1966). The irregular gaits cannot exclusively be characterized by walking speed because walk and wave gaits occurred at the same average speed as irregular gaits (Fig. 10).

First instar stick insect amputees change their regular tripod gait into a gait similar to gait II (Graham, 1976), a walk gait (the corresponding figure in Graham's paper actually shows a wave gait). Graham did not report unstable situations for instars. However, we can assume that the walking system becomes less rigidly coordinated in the course of ontogenesis. In this context,

it is interesting that the model Walknet, a biologically inspired network to control six-legged locomotion that is based on the previously mentioned rules for coordination (Dürr et al., 2004) (see also Cruse, 1990), failed to produce a coordinated walking pattern after 'amputation' of both middle legs (Schilling et al., 2007). A stable coordination was regained after introducing a rule that prevents a front leg swing when the ipsilateral hindleg is swinging. If such a mechanism is present in the adult stick insect, it appears to be weakly developed.

Our experiments indicate that in stick insects the presence of middle legs is important for the organization of a regular functional activity pattern in the hindlegs as seen in intact animals and front leg amputees. The mechanisms for such influences are unclear, especially as Borgmann and colleagues (Borgmann et al., 2007) have shown that in a single middle leg preparation the stepping middle leg is not able to induce rhythmic alternating activity in either hindleg or front leg protractor and retractor motoneurons (Borgmann et al., 2007). Thus, under their experimental conditions, the effects of middle leg stepping on the adjacent ipsilateral legs were quite weak. In contrast, single front leg walking does induce rhythmic activity in middle leg protractor and retractor motoneurons and is even able to produce in-phase coupling of pharmacologically induced alternating activity in hindleg protractor and retractor motoneurons (Borgmann et al., 2009). While the effect of front leg walking on the hindlegs is weak, the effect on middle leg protractor/retractor motoneuron activity is quite strong (Ludwar et al., 2005; Borgmann et al., 2007). Again, such a strong effect is not seen in our

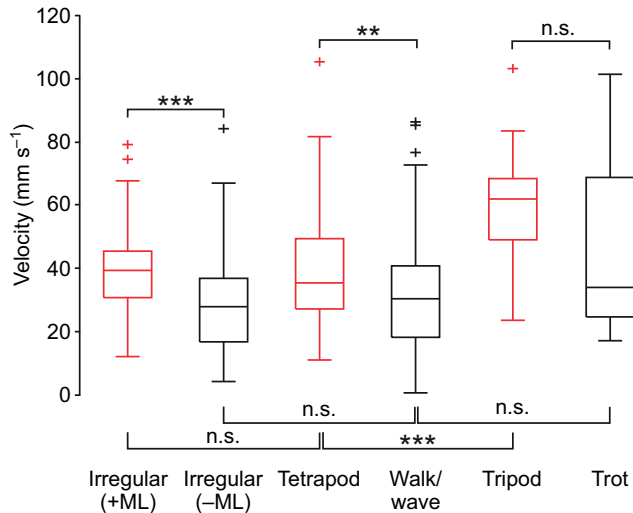


Fig. 10. Walking velocities in intact animals (red, +ML) and middle leg amputees (black, -ML). Box-whisker plots are shown for irregular, tetrapod, walk/wave, tripod and trot gaits (** $P < 0.01$, *** $P < 0.001$).

experiments, where the middle legs in hindleg amputees show a more regular pattern than the front legs. In addition, the in-phase coupling of retractor and protractor motoneurons in ipsilateral legs observed by Borgmann and colleagues (Borgmann et al., 2007; Borgmann et al., 2009) does not match our data. However, the importance of local load signals and their ability to overcome intersegmental entrainment has already been mentioned and may also be responsible for our observations in middle leg amputees. In a stick insect walking on a treadmill, the impact of such load signals is likely to be the cause of the $\sim 180^\circ$ phase shift in protractor and retractor activity after ground contact was regained by mounting a peg leg to a partly amputated middle leg (Wendler, 1965). Middle leg afferents appear to be essential not only for an adequate middle leg phasing but also for intersegmental coordination. For a proper coordination of ipsilateral legs, phasic afferent input induced by the normal motions of the middle legs appears to be important because in tethered stick insects walking on a treadmill, ipsilateral front leg and hindleg movements are not phase locked to one another when the middle legs rest on a platform (Wendler, 1965). Such uncoupling of front legs and hindlegs might destabilize walking in untethered middle leg amputees.

Gaits in amputees and inter-leg control

Our experiments corroborate the observation that, after amputation, arthropods generally adopt a functional gait with phase relationships different from those for six-legged locomotion. Amputees in our experiments often adopted the wave gait, which was rarely observed in intact animals. Such adaptivity has been reported for spiders (Wilson, 1967; Foelix, 1996), cockroaches (Hughes, 1957; Delcomyn, 1971), stick insects (von Buddenbrock, 1921; von Holst, 1943; Wendler, 1965; Graham, 1977) and other arthropods (see review by Wilson, 1966). The adaptations provide stability and are probable means of reducing energy expenditure, like gait changes in intact animals that adapt to changing speed (Alexander, 1989; Nishii, 2000). A rare example of a non-adaptive behaviour is escape running in cockroaches that carry an extra load. The startled animals use a tripod gait that is not functional because it does not compensate for the increased load (Quimby et al., 2005).

At first glance, the notion that inter-leg coordination depends on sensory feedback appears to be trivial. In locusts, it has been shown that signals from leg sense organs are largely conveyed by intersegmental interneurons to other ganglia and only rarely do sense organs have intersegmental projections (Hustert, 1978). The intersegmental interneurons can be descending (Laurent, 1987; Laurent and Burrows, 1989a; Laurent and Burrows, 1989b) or ascending (Laurent and Burrows, 1988). Some mesothoracic intersegmental interneurons make connections with ipsilateral metathoracic non-spiking interneurons and motoneurons (Laurent and Burrows, 1989b). However, the role of sense organs in inter-leg coordination is poorly understood (for review, see Büschges and Gruhn, 2007). For example, the neuronal basis of the coordination rules identified by Cruse and co-workers (Cruse, 1990; Dürr et al., 2004) is still largely unknown. So far, only a few interneurons in the stick insect have been identified that could mediate intersegmental coordination between middle legs and hindlegs (Brunn and Dean, 1994). This lack of knowledge is in sharp contrast to the wealth of information we have for local feedback control of a single leg stepping cycle (for review, see Büschges, 2005; Büschges and Gruhn, 2007). Cruse (Cruse, 1985a; Cruse, 1985b) provides evidence that swing phase starts upon a small initial forward movement of the leg followed by a decrease in load, which also reinforces swing phase muscle activity as shown by Akay and colleagues (Akay et al., 2001; Akay et al., 2004). Such mechanisms are also effective in inter-leg control. In cockroaches, triggering activity in campaniform sensilla of the middle leg by mechanical action of the hindleg could facilitate the onset of swing in the middle leg through local reflex effects (Zill et al., 2009). Such an emergent mechanism of inter-leg control that results from mechanical forces should be effective whenever legs are mechanically coupled through the substrate. Interestingly, however, lasting stable functional coordination of leg movements was rarely achieved by middle leg amputees, but was often found in front leg and hindleg amputees (we do not consider multiple stepping in front legs). Therefore, inter-leg coordination appears to depend largely on intersegmental neural pathways that are most effective between adjacent legs.

CONCLUSIONS

(1) Front legs have a special role during walking as they show multiple stepping, independent of the walking cycles of the adjacent legs. At the same time, coordination in middle legs and hindlegs is organized in gaits typical for quadrupeds. Inter-segmental information from the front legs is not necessary to coordinate and stabilize these patterns. However, front legs can be coupled to the locomotor system and then regular hexapedal walking is generated. This would correspond to a strengthening of the inter-segmental connections at least between the front and middle legs. (2) Afferent information from the middle legs seems to be necessary to produce regular stepping in the hindlegs. This assumption implies a special role for middle leg afferent signals in inter-leg coordination. (3) Hindleg afferent signals, in contrast, do not seem to be necessary for middle legs to produce regular stepping.

The different functionality of the legs and the resulting flexibility of the walking system need therefore to be considered in modelling studies of insect locomotion.

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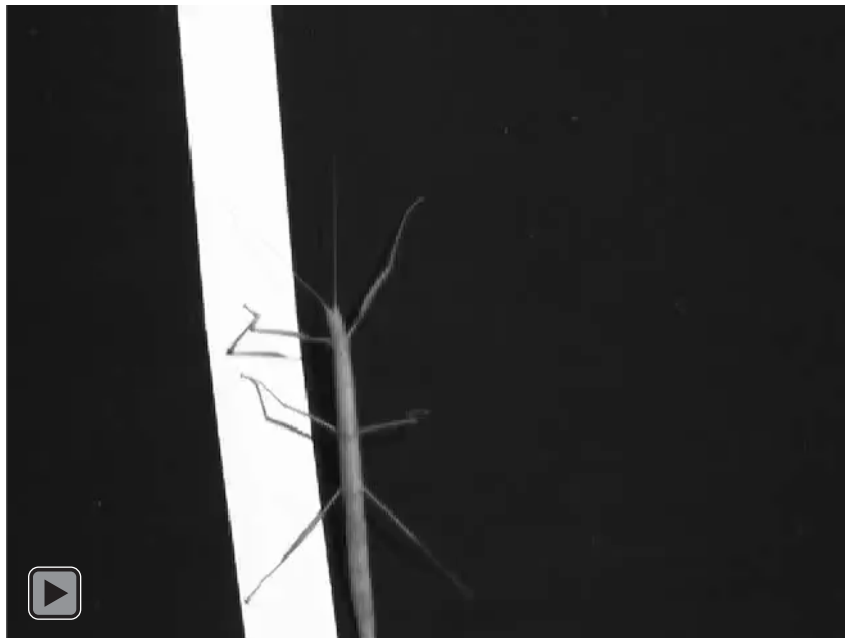
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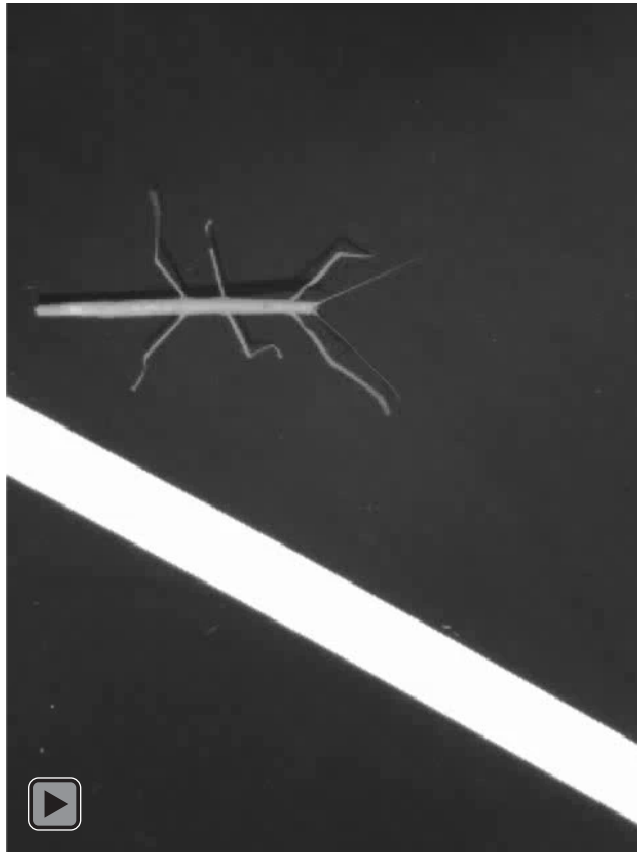
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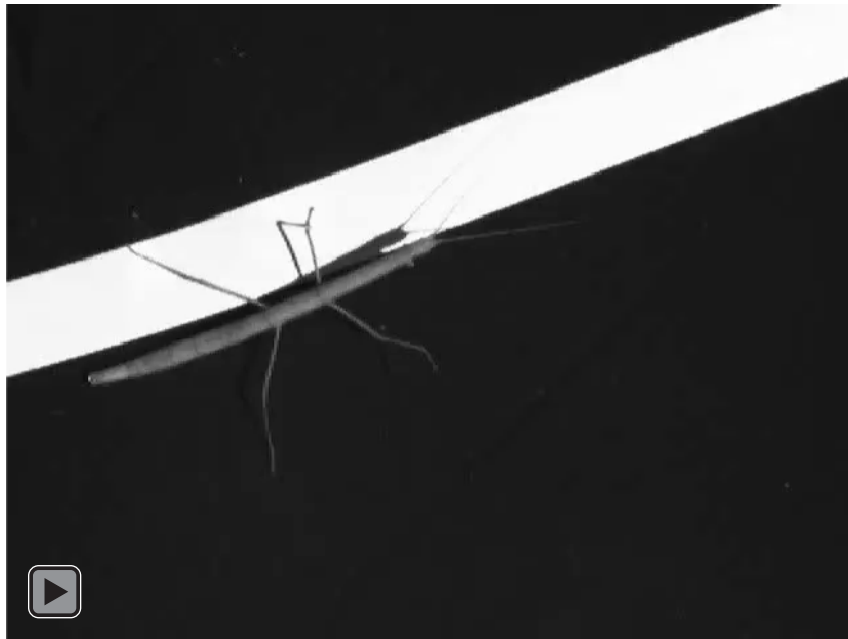
Movie 1. An example of an intact adult stick insect walking in a tetrapod gait.



Movie 2. An example of an intact adult stick insect walking in a tripod gait.



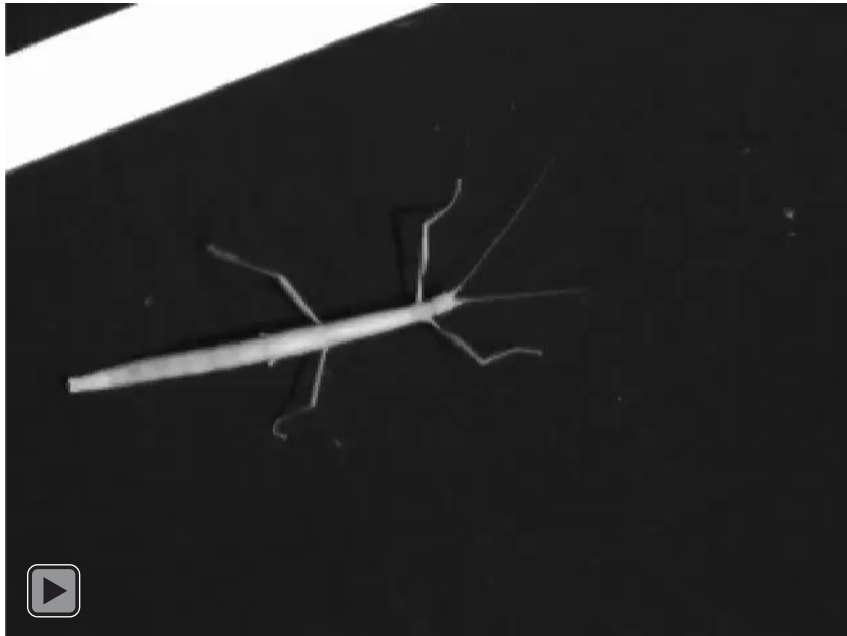
Movie 3. An example of an intact adult stick insect walking in an irregular gait.



Movie 4. An example of an intact adult stick insect walking with amputated front legs.



Movie 5. An example of an intact adult stick insect walking with amputated middle legs.



Movie 6. An example of an intact adult stick insect walking with amputated hindlegs.